SF 298 MASTER COPY KEEP THIS COPY FOR REPRODUCTION PURPOSES

REPORT DOCUMENTATION PAGE

Form Approved
OMB NO. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 30, 1997	Final Progress 6/20/94 - 6/19/97

4. TITLE AND SUBTITLE

Enzyme Design for Nonaqueous Solvents

5. FUNDING NUMBERS

DAAH04-94-G-0178

6. AUTHOR(S)

Jonathan S. Dordick[1]
Douglas S. Clark[2]

7. PERFORMING ORGANIZATION NAMES(S) AND ADDRESS(ES)

[1]Department of Chemical & Biochemical Engineering, 125 Chemistry Bldg, The University of Iowa, Iowa City, IA 52242. [2]Department of Chemical Engineering, University of California at Berkeley, Berkeley, CA 94720-9989.

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Research Office
P.O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

ARO 33139.6-LS

11. SUPPLEMENTARY NOTES

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision, unless so designated by other documentation.

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited.

12 b. DISTRIBUTION CODE

DTIC QUALITY INSPECTED 2

13. ABSTRACT *(Maximum 200 words)*

From a biotechnological perspective there are many advantages of employing enzymes in organic as opposed to aqueous media. While there is now little question that enzymes can function in organic media, reaction rates are typically quite low. Relatively few kinetic studies have been carried out, and detailed investigations of enzyme structure and dynamics are fewer still. The overall goal of this ARO-sponsored research has been to identify the factors that govern enzyme activity and specificity in organic solvents, and apply this knowledge in the design of enzyme catalysts with optimal (i.e., aqueous-like) activity in nonaqueous media. In addition, this research set out to expand on our recent success in activating enzymes for use in organic solvents - in some cases activations of greater than 1000-fold have been achieved - and to understand fundamentally the factors that govern enzyme activity and enzyme activation in organic solvents. The knowledge gained in this investigation can be applied to a more general design of enzyme catalysts with optimal (i.e., aqueous-like) activity and tailored selectivity in nonaqueous media.

14. SUBJECT TERMS	15. NUMBER IF PAGES
enzymes in organic solvents, enzyme activation in dehydrated media, solvent effects on enzyme mechanisms, subtilisin catalysis	9
	16. PRICE CODE

17. SECURITY CLASSIFICATION OR REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UL

GENERAL INSTRUCTIONS FOR COMPLETING SF 298

The Report Documentation Page (RDP) is used in announcing and cataloging reports. It is important that this information be consistent with the rest of the report, particularly the cover and title page. instructions for filling in each block of the form follow. It is important to ***stay within the lines*** to meet ***optical scanning requirements.***

Block 1. Agency Use Only *(Leave blank)*

Block 2. Report Date. Full publication date including day, month, and year, if available (e.g. 1 Jan 88). Must cite at least year.

Block 3. Type of Report and Dates Covered. State whether report is interim, final, etc. If applicable, enter inclusive report dates (e.g. 10 Jun 87 - 30 Jun 88).

Block 4. Title and Subtitle. A title is taken from the part of the report that provides the most meaningful and complete information. When a report is prepared in more than one volume, repeat the primary title, add volume number, and include subtitle for the specific volume. On classified documents enter the title classification in parentheses.

Block 5. Funding Numbers. To include contract and grant numbers; may include program element number(s), project number(s), task number(s), and work unit number(s). Use the following labels:

C	- Contract	**PR**	- Project
G	- Grant	**TA**	- Task
PE	- Program Element	**WU**	- Work Unit Accession No.

Block 6. Author(s). Name(s) of person(s) responsible for writing the report, performing the research, or credited with the content of the report. If editor or compiler, this should follow the name(s).

Block 7. Performing Organization Name(s) and Address(es). Self-explanatory.

Block 8. Performing Organization Report Number. Enter the unique alphanumeric report number(s) assigned by the organization performing the report.

Block 9. Sponsoring/Monitoring Agency Name(s) and Address(es). Self-explanatory.

Block 10. Sponsoring/Monitoring Agency Report Number. *(If known)*

Block 11. Supplementary Notes. Enter information not included elsewhere such as; prepared in cooperation with...; Trans. of...; To be published in.... When a report is revised, include a statement whether the new report supersedes or supplements the older report.

Block 12a. Distribution/Availability Statement. Denotes public availability or limitations. Cite any availability to the public. Enter additional limitations or special markings in all capitals (e.g. NORFORN, REL, ITAR).

- **DOD** - See DoDD 4230.25, "Distribution Statements on Technical Documents."
- **DOE** - See authorities.
- **NASA** - See Handbook NHB 2200.2.
- **NTIS** - Leave blank.

Block 12b. Distribution Code.

- **DOD** - Leave blank
- **DOE** - Enter DOE distribution categories from the Standard Distribution for Unclassified Scientific and Technical Reports
- **NASA** - Leave blank.
- **NTIS** - Leave blank.

Block 13. Abstract. Include a brief *(Maximum 200 words)* factual summary of the most significant information contained in the report.

Block 14. Subject Terms. Keywords or phrases identifying major subjects in the report.

Block 15. Number of Pages. Enter the total number of pages.

Block 16. Price Code. Enter appropriate price code *(NTIS only).*

Block 17. - 19. Security Classifications. Self-explanatory. Enter U.S. Security Classification in accordance with U.S. Security Regulations (i.e., UNCLASSIFIED). If form contains classified information, stamp classification on the top and bottom of the page.

Block 20. Limitation of Abstract. This block must be completed to assign a limitation to the abstract. Enter either UL (unlimited) or SAR (same as report). An entry in this block is necessary if the abstract is to be limited. If blank, the abstract is assumed to be unlimited.

ENZYME DESIGN FOR NONAQUEOUS SOLVENTS

JONATHAN S. DORDICK, PH.D. AND DOUGLAS S. CLARK, PH.D.

OCTOBER 30, 1997

U.S. ARMY RESEARCH OFFICE

CONTRACT/GRANT NUMBER: DAAH04-94-G-0307

UNIVERSITY OF IOWA, DEPARTMENT OF CHEMICAL AND BIOCHEMICAL ENGINEERING

AND

UNIVERSITY OF CALIFORNIA, BERKELEY, DEPARTMENT OF CHEMICAL ENGINEERING

1. FORWARD

From a biotechnological perspective there are many advantages of employing enzymes in organic as opposed to aqueous media. While there is now little question that enzymes can function in organic media, reaction rates are typically quite low. Relatively few kinetic studies have been carried out, and detailed investigations of enzyme structure and dynamics are fewer still. The overall goal of this ARO-sponsored research has been to identify the factors that govern enzyme activity and specificity in organic solvents, and apply this knowledge in the design of enzyme catalysts with optimal (i.e., aqueous-like) activity in nonaqueous media. In addition, this research set out to expand on our recent success in activating enzymes for use in organic solvents - in some cases activations of greater than 1000-fold have been achieved - and to understand fundamentally the factors that govern enzyme activity and enzyme activation in organic solvents. The knowledge gained in this investigation can be applied to a more general design of enzyme catalysts with optimal (i.e., aqueous-like) activity and tailored selectivity in nonaqueous media.

2. TABLE OF CONTENTS (IF MORE THAN 10 PAGES)

Not Applicable

3. LIST OF APPENDIXES, ILLUSTRATIONS AND TABLES

Not Applicable

4. BODY OF REPORT

A. STATEMENT OF THE PROBLEM STUDIED

The goals of this ARO-sponsored research were as follows:

1. **To investigate the effect of solvent properties (e.g., hydrophobicity, polarity, hydration) on enzyme activity and specificity**.

2. **To tailor and improve enzyme activity in organic solvents by a) protein engineering, b) solvent engineering, and c) catalyst engineering (e.g., use of supports, additives, and natural stabilizers)**.

3. **To control enzyme specificity by protein and solvent engineering**.

4. **To develop rational and predictive guidelines for biocatalyst optimization in organic solvents.**

Much of this work was performed using the serine proteases subtilisin (Carlsberg and BPN') and α-chymotrypsin, as well as the non-serine protease, thermolysin. Instead of a broad summary of the results and impact of this work, we provide a complete compilation of the abstracts of publications that have resulted from this research. A detailed bibliography of these publications is given in Section C below.

B. SUMMARY OF PUBLISHED RESULTS

1) Khmelnitsky, Yu.L., Welch, S.H., Clark, D.S., and Dordick, J.S. (1994), "Salts Dramatically Enhance Activity of Enzymes Suspended in Organic Solvents", J. Am. Chem. Soc. **116**, 2647-2648.

The catalytic efficiencies (k_{cat}/K_m) of subtilisin Carlsberg and α-chymotrypsin in anhydrous organic solvents are dramatically increased when each enzyme is lyophilized in the presence of excess salts. For example, a biocatalyst powder containing 98% (w/w) KCl, and 1% (w/w/) each of phosphate buffer and subtilisin was 3,750-times more active in n-hexane for the transesterification reaction between N-Ac-L-Phe-OEt and 1-propanol than the enzyme prepared without KCl. This activation was primarily due to a large increase in the catalytic turnover (k_{cat}) and provides for catalytic efficiencies in n-hexane to within an order of magnitude of that obtained for hydrolytic reactions in aqueous solutions. The activation was also observed in other organic solvents as well as with other salts indicating that the effect may be general. Enzymatic catalysis in the gas phase was not dramatically affected by the presence of salt in the lyophilized mixture suggesting that salt does not act as a lyoprotectant, but rather protects the enzyme from direct interaction (and inactivation) by the organic solvent. We hypothesize that a highly polar salt matrix is formed that excludes direct solvent contact with the enzyme and helps to maintain the native structure of the enzyme.

2) Paradkar, V.M. and Dordick, J.S. (1994), "Aqueous-Like Activity of α-Chymotrypsin Dissolved in Nearly Anhydrous Organic Solvents", J. Am. Chem. Soc. **116**, 5009-5010.

α–Chymotrypsin (CT) is easily extracted from an aqueous phase into isooctane containing a low concentration of surfactant (≤ 2 mM AOT) via the formation of ion pairs between the protein and the surfactant. The low surfactant concentration eliminates the formation of reversed micelles. Subsequent drying of the enzyme-containing isooctane phase results in an extensively dehydrated soluble enzyme in isooctane. This soluble enzyme preparation can then be dissolved in a wide variety of organic solvents. Soluble CT is highly active in isooctane, with a k_{cat}/K_m for the transesterification of Ac-L-Phe-OEt with n-propanol of 3,020 $M^{-1}s^{-1}$ which is ca. 15% of that in aqueous buffer for Ac-L-Phe-OEt hydrolysis. The soluble enzyme is ca. 2,500-fold more active than CT suspended in isooctane. Spectral analysis of soluble CT in organic solvents do not reveal any significant changes as compared to soluble CT in water, hence the soluble enzyme retains its native secondary and tertiary structure. The highly active soluble CT was used for thermodynamically-controlled peptide synthesis resulting in efficient synthesis of dipeptides in yields approaching 100%. In addition to CT, subtilisin Carlsberg was dissolved in isooctane in a similar manner and shows a value of k_{cat}/K_m for the transesterification of Ac-L-Phe-OEt and n-propanol of 68% of that for hydrolysis in aqueous buffer.

3) A. M. Blinkovsky, Yu. L. Khmelnitsky, and J. S. Dordick (1994), "Organosoluble Enzyme-Polymer Complexes: A Novel Type of Biocatalyst for Nonaqueous Media", Biotechnol. Techniques **8**, 33-38.

A novel type of organosoluble biocatalyst, representing a non-covalent complex of enzyme with a sugar-based amphiphilic polymer is described. The subtilisin Carlsberg-palmitoyl poly(sucrose acrylate) complex was found to be soluble and catalytically active in a number of organic solvents of different nature.

4) P. P. Wangikar, D. S. Clark, and J. S. Dordick (1995), "Probing Enzymic Transition State Hydrophobicities ", Biochemistry **34**, 12302-12310.

Hydrophobic interactions are important in numerous biological processes; however, the nature and extent of hydrophobic interactions in nonaqueous enzymology remain poorly defined. We have estimated the free energies of enzyme-substrate hydrophobic interactions for a model reaction catalyzed by subtilisin BPN' (from *Bacillus amyloliquefaciens*). Transition state stabilization of subtilisin in water has contributions from both ground state destabilization of hydrophobic substrates in water and intrinsic enzyme-substrate hydrophobic interactions. Both contributions are evident even in hydrophobic organic solvents, and can be modified by protein engineering of the enzyme's binding site as well as by changing the hydrophobicity of the reaction medium. We have also developed a method to estimate the hydrophobicity of the enzymic transition state involving systematic variation of the substrate and solvent hydrophobicities. The observed binding pocket hydrophobicities were directly affected by replacing the Gly_{166} residue, located at the back of the hydrophobic S_1 binding pocket of subtilisin BPN', with more hydrophobic amino acids such as alanine and valine. Thus, the observed S_1 binding pocket hydrophobicities of the wild-type, G166A, and G166V mutants were measured to be 1.2, 1.8, and 2.6 log P units, respectively. Our method of calculating effective binding pocket hydrophobicity was found to be applicable to other enzymes including horseradish peroxidase and α-chymotrypsin. The measurements of the binding pocket hydrophobicities have significant implications toward tailoring enzyme function in aqueous as well as nonaqueous media.

5) V. M. Suzawa, Yu. L. Khmelnitsky, L. Giarto, J. S. Dordick, and D. S. Clark (1995), "Suspended and Immobilized Chymotrypsin in Organic Media: Structure-Function Relationships Revealed by ESR Spectroscopy", J. Am. Chem. Soc. **117**, 8435-8440.

Comparing the behavior of freely suspended and immobilized enzyme in organic media with low water contents can yield insights into interactions among the biocatalyst, solvent, and support that influence protein structure and function. Immobilized chymotrypsin has higher activity than the suspended enzyme (by one to two orders of magnitude) in anhydrous organic solvents ranging from nonpolar n-octane to polar acetonitrile. In anhydrous tetrahydrofuran (THF), glass-adsorbed chymotrypsin is ca. ten-times more active than the suspended enzyme, and electron spin resonance (ESR) spectra of an active-site spin label reveal greater local flexibility. Upon adding up to 0.5% v/v water, increased catalytic efficiency of the immobilized enzyme is accompanied by a sharp rise in active-site polarity but no apparent change in active-site conformation or dynamics. Under the same conditions the activity of the suspended enzyme also increases; however, the active-site polarity remains nearly constant while the spin label reflects increasing molecular flexibility. For both preparations, further changes in protein structure occur as enzyme activity increases with 0.5 to 7% v/v added water. Computer simulations of the room-temperature ESR spectra suggest that different initial conformational states contribute to the different behavior of the two enzyme systems over the entire range of added water. These findings show that the structural properties of suspended and immobilized enzyme can differ markedly and that these differences are important to enzyme activity in organic media.

6) P. P. Wangikar, D. Carmichael, D. S. Clark, and J. S. Dordick (1995), "Active Site Titration of Serine Proteases in Organic Solvents", Biotechnol. Bioeng. **50**, 329-335.

Calculation of kinetic constants of an enzymatic reaction in organic solvents requires knowledge of the functional active-site concentration in organic solvents, and this can be significantly different than that in water. An experimental method for active-site titration of serine proteases in organic media has been developed based on the kinetics of inhibition by phenylmethanesulfonyl fluoride (PMSF), a serine-specific inhibitor (or suicide substrate). This kinetic approach is fundamentally different from other techniques that require complete titration of all accessible enzyme active sites. This active site titration method was applied to subtilisins BPN' and Carlsberg, and α-chymotrypsin, and resulted in fractions of active sites that ranged from 8 to 62% (of the fraction active in water) depending on the enzyme, the method of enzyme preparation, and the organic solvent used. The active site concentration of subtilisin BPN' and Carlsberg increased with increasing hydrophobicity of the solvent, and with increasing solvent hydration in tetrahydrofuran. The dependence of the fraction of active sites on the nature of the organic solvent appears to be governed largely by solvent-induced inactivation caused by direct interaction of a hydrophilic solvent with the enzyme.

7) J. O. Rich, B. A. Bedell, and J. S. Dordick (1995), "Controling Enzyme-Catalyzed Regioselectivity in Sugar Ester Synthesis", Biotechnol. Bioeng. **45**, 426-434.

The rational control over enzyme-catalyzed regioselectivity has been studied using sucrose acylation by vinyl esters in organic media as a model. Subtilisins BPN' and Carlsberg preferentially acylate at the 1'-hydroxyl of sucrose with some acylation observed at the 6-hydroxyl. The preference for the 1'-hydroxyl is strongly affected by the hydrophobicity of the organic solvent and the chain length of the vinyl ester. Increasingly hydrophobic solvents and longer chain lengths lower the favorable formation of the 1'-acylation and improve 6-acylation. Molecular modeling of sucrose in the binding pocket of subtilisin BPN' shows that the 1'-acylation is favored in solvents that can solvate sugars (such as pyridine) as the glucose moiety is exposed to the medium, whereas 6-acylation leaves the entire sucrose molecule buried within the enzyme's binding pocket. Thus, 1'-acylation is sterically more favorable than 6-acylation. Increasingly hydrophobic solvents affect regioselectivity by changing the degree of solvation of the glucose moiety in the medium and forcing the sucrose 1'-ester completely into the binding pocket. In a related modeling, the vinyl ester chain length was shown to modulate regioselectivity by controlling the bond angles between the resulting acyl- enzymes and the sucrose thereby affecting the positioning of the sucrose in the binding pocket of subtilisin BPN'. This study shows that control over enzymic regioselectivity can be achieved by rational choices of substrate and solvent.

8) P. P. Wangikar, P. C. Michels, D. S. Clark, and J. S. Dordick (1997), "Structure and Function of Subtilisin BPN' Solubilized in Organic Solvents", J. Am. Chem. Soc. **119**, 70-76.

Enzyme structure and function have been studied for subtilisin BPN' solubilized in organic solvents by ion pairing with low concentrations of an anionic surfactant (Aerosol OT) in the absence of reversed micelles. Soluble subtilisin shows strikingly different behavior in octane and tetrahydrofuran (THF). In octane, the k_{cat}/K_m for the transesterification of N-Acetyl L-Phenylalanine Ethyl Ester (APEE) is 370 $M^{-1}s^{-1}$, within one order of magnitude of the enzyme's hydrolytic activity in water. Moreover, the

observed half-life of the soluble enzyme in octane is nearly three orders of magnitude greater than in water, presumably due to the absence of autolysis in the organic solvent. In contrast, the catalytic efficiency of the enzyme dissolved in the polar solvent THF is 0.04 $M^{-1}s^{-1}$, and the enzyme loses 99% of its activity within 10 min. Comparable enzyme inactivation could also be observed in octane, but only at elevated temperatures such as 70°C. Therefore, the mechanisms of deactivation of the soluble enzyme were investigated in both octane and THF. Kinetic and spectroscopic (CD and EPR) studies support the existence of multiple inactive forms of the soluble enzyme in THF at 25°C and in octane at 70°C. Notably, in both cases a denatured form can be renatured in anhydrous octane at 25°C, the first demonstration of enzyme renaturation in a bulk organic solvent. A model explaining the THF- and thermally-induced inactivation processes of soluble subtilisin BPN' is proposed, and the apparent reasons for the exceptionally high activity and stability of the soluble enzyme in octane are discussed.

9) J. O. Rich and J. S. Dordick (1997), "ControllingSubtilisin Activity and Selectivity in Organic Media by Imprinting with Nucleophilic Substrates", J. Am. Chem. Soc. **119**, 3245-3252.

The activity and substrate specificity of subtilisin-catalyzed acylation of nucleosides in organic solvents can be controlled by lyophilizing the enzyme from an aqueous solution containing the substrate. This "molecular imprinting" technique was examined using thymidine as a model nucleoside, and the resulting subtilisin preparation was up to 50-fold more reactive toward thymidine acylation in nearly anhydrous tetrahydrofuran than subtilisin lyophilized from aqueous buffer in the absence of the nucleoside. Although several compounds, including thymine and ribose, improved the rate of thymidine acylation, the thymidine-imprinted enzyme was the most efficient catalyst for this reaction. Furthermore, it was possible to alter the substrate selectivity of subtilisin by lyophilizing the enzyme in the presence of a different nucleophilic substrate. For example, imprinting made possible the discrimination between structurally different (i.e., sucrose versus thymidine) as well as structurally similar (i.e., thymidine versus deoxyadenosine) nucleophiles. Molecular modeling studies of the interaction of thymidine or the unrelated sucrose with subtilisin revealed that structural changes upon imprinting in the serine protease's catalytic triad may be responsible for the observed activation and selectivity changes. Further use of molecular dynamics indicated that structural changes in the catalytic triad occur during imprinting, and that these changes may be the major factor that contributes to imprinting-induced substrate selectivity. This contrasts with the previously held notion that imprinting influences mainly substrate binding.

10) P. C. Michels, J. S. Dordick, and D. S. Clark (1997), "Dipole Formation and Solvent Electrostriction in Subtilisin Catalysis", J. Am. Chem. Soc. **119**, 9331-9335.

The transition state for subtilisin-catalyzed transesterification was probed by high-pressure kinetic studies in solvents spanning a wide range of dielectric constants. The electrostatic model of Kirkwood described the solvent effects and gave a lower limit of 31±1.5 Debye for the dipole moment of the transition state. This value remained constant in a wide range of polar and apolar solvents, indicating that the catalytic triad of subtilisin is remarkably robust. Despite the highly polar transition state, substantial rate enhancements relative to the uncatalyzed reaction were measured in highly apolar solvents such as hexane; this is the first report of such an extreme disparity between transition-state and solvent polarities. Moreover, the solvent dependence of the activation volume implies a low effective dielectric of the polypeptide chain in the active site, and substantial penetration of the active site by solvent. Kirkwood's model

was also used to quantify the effect of an active-site mutation on the transition-state dipole moment. These results illustrate that the electrostatic model combined with high-pressure kinetics can provide unique information on the basic properties of enzyme reaction processes, and can be useful in predicting solvent effects on enzyme reaction rates.

11) C. S. Lee, M. Haake, M. T. Ru, J. S. Dordick, J. A. Reimer, and D. S. Clark (1997), "A Multinuclear NMR Study of Enzyme Hydration in an Organic Solvent", Biotechnol. Bioeng. (in press).

Multinuclear NMR spectroscopy has been used to study water bound to subtilisin Carlsberg suspended in tetrahydrofuran (THF), with the water itself employed as a probe of the hydration layer's physicochemical and dynamic characteristics. The presence of the enzyme did not affect the intensity, chemical shift or linewidth of water (up to 8% v/v) added to THF, as measured by ^{17}O- and ^{2}H-NMR. This finding suggests that hydration of subtilisin can be described by a three-state model that includes tightly bound, loosely bound, and free water. Solid-state ^{2}H-NMR spectra of enzyme-bound D_2O support the existence of a non-exchanging population of tightly bound water. An important implication is that the loosely-bound water is the same as free water from an NMR viewpoint. This loosely-bound water must also be the water responsible for the large increase in catalytic activity observed in previous hydration studies.

12) B. A. Bedell, V. V. Mozhaev, D. S. Clark, and J. S. Dordick (1997), "Testing for Diffusion Limitations in Salt-Activated Enzyme Catalysts Operating in Organic Solvents", Biotechnol. Bioeng. (in press).

The dramatic activation of serine proteases in nonaqueous media resulting from lyophilizing in the presence of KCl, first reported by Khmelnitsky et al. (1994), is shown to be unrelated to relaxation of potential substrate diffusional limitations. Specifically, lyophilizing subtilisin Carlsberg in the presence of KCl and phosphate buffer in different proportions, ranging from 99% (w/w) enzyme to 1% (w/w) enzyme in the final lyophilized solids, resulted in biocatalyst preparations that were not influenced by substrate diffusion, and thereby retained their kinetically-controled properties. This result was evident through use of a classical analysis whereby the initial rates of catalysis as a function of active enzyme in the biocatalyst preparation, normalized per constant volume of catalyst material, were measured for all biocatalyst preparations studied. Plots of initial reaction rates as a function of the percent of active subtilisin in the biocatalyst preparations were linear for all biocatalyst preparations. Thus, enzyme activation (reported by Khmelnitsky et al. (1994) to be as high as 3750-fold in hexane for the transesterification of N-Ac-L-Phe-OEt with n-PrOH) is a manifestation of intrinsic enzyme activation and not relaxation of diffusional limitations resulting from diluted enzyme preparations. Similar activation is reported for thermolysin, a non-serine protease, thereby demonstrating that enzyme activation due to lyophilizing in the presence of KCl may be a general phenomenon.

C. LIST OF PUBLICATIONS

1) Khmelnitsky, Yu.L., Welch, S.H., Clark, D.S., and Dordick, J.S. (1994), "Salts Dramatically Enhance Activity of Enzymes Suspended in Organic Solvents", J. Am. Chem. Soc. **116**, 2647-2648.

2) Paradkar, V.M. and Dordick, J.S. (1994), "Aqueous-Like Activity of α-Chymotrypsin Dissolved in Nearly Anhydrous Organic Solvents", J. Am. Chem. Soc. **116**, 5009-5010.

3) A. M. Blinkovsky, Yu. L. Khmelnitsky, and J. S. Dordick (1994), "Organosoluble Enzyme-Polymer Complexes: A Novel Type of Biocatalyst for Nonaqueous Media", Biotechnol. Techniques **8**, 33-38.

4) P. P. Wangikar, D. S. Clark, and J. S. Dordick (1995), "Probing Enzymic Transition State Hydrophobicities ", Biochemistry **34**, 12302-12310.

5) V. M. Suzawa, Yu. L. Khmelnitsky, L. Giarto, J. S. Dordick, and D. S. Clark (1995), "Suspended and Immobilized Chymotrypsin in Organic Media: Structure-Function Relationships Revealed by ESR Spectroscopy", J. Am. Chem. Soc. **117**, 8435-8440.

6) P. P. Wangikar, D. Carmichael, D. S. Clark, and J. S. Dordick (1995), "Active Site Titration of Serine Proteases in Organic Solvents", Biotechnol. Bioeng. **50**, 329-335.

7) J. O. Rich, B. A. Bedell, and J. S. Dordick (1995), "Controling Enzyme-Catalyzed Regioselectivity in Sugar Ester Synthesis", Biotechnol. Bioeng. **45**, 426-434.

8) P. P. Wangikar, P. C. Michels, D. S. Clark, and J. S. Dordick (1997), "Structure and Function of Subtilisin BPN' Solubilized in Organic Solvents", J. Am. Chem. Soc. **119**, 70-76.

9) J. O. Rich and J. S. Dordick (1997), "ControllingSubtilisin Activity and Selectivity in Organic Media by Imprinting with Nucleophilic Substrates", J. Am. Chem. Soc. **119**, 3245-3252.

10) P. C. Michels, J. S. Dordick, and D. S. Clark (1997), "Dipole Formation and Solvent Electrostriction in Subtilisin Catalysis", J. Am. Chem. Soc. **119**, 9331-9335.

11) C. S. Lee, M. Haake, M. T. Ru, J. S. Dordick, J. A. Reimer, and D. S. Clark (1997), "A Multinuclear NMR Study of Enzyme Hydration in an Organic Solvent", Biotechnol. Bioeng. (in press).

12) B. A. Bedell, V. V. Mozhaev, D. S. Clark, and J. S. Dordick (1997), "Testing for Diffusion Limitations in Salt-Activated Enzyme Catalysts Operating in Organic Solvents", Biotechnol. Bioeng. (in press).

D. LIST OF PARTICIPATING SCIENTIFIC PERSONNEL AND ADVANCED DEGREE EARNED

Yuri Khmelnitsky - Postdoctoral scientist (1992-1994), U. Iowa
Jungbae Kim - Ph.D. awarded, 5/95, U. Iowa
Pramod Wangikar - Ph.D. awarded, 5/95, U. Iowa
Valerie Suzawa - Ph.D. awarded 8/95, U. C. Berkeley
Joseph Rich - Ph.D. awarded 8/96, U. Iowa
Bruce Bedell - M.S. Candidate (1995 - present), U. Iowa
Peter Michels - Ph.D. awarded 10/96, U. C. Berkeley
Christopher Lee - M.S. awarded 12/96, U. C. Berkeley
Vadim Mozhaev - Postdoctoral scientist (1996 - present), U. Iowa
Michael Ru - Ph.D. Candidate (1996 - present), U. C. Berkeley

5. REPORT OF INVENTIONS ONLY

"Reacting an Enzyme in a Nonaqueous Solvent by Adding a Lyophilizate of Enzyme and Salt to the Solvent", J. S. Dordick, D. S. Clark, and Y. L.Khmelnitsky, U.S. Patent 5,449,613.

6. BIBLIOGRAPHY

Not Applicable

7. APPENDICES

Not Applicable